

Fungal Tools for the Degradation of Endocrine Disrupting Compounds

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“Wer ins Ausland geht, führt keinen Krieg.” Jan Christoph Wiechmann, Journalist

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Abstract

Endocrine disrupting compounds are synthetic or natural compounds that mimic the action of hormones and thus disrupt or alter functions of the endocrine system usually through direct interactions with nuclear receptors. The main objective of this work was to develop strategies of how to degrade endocrine disrupting compounds and how to monitor the removal of the endocrine disrupting effect with focus on the estrogenic compound bisphenol A and on the virilizing compound tributyltin.

Bisphenol A is used as plasticizer of polycarbonate plastics, which are used for food packaging, coatings of metal cans and baby bottles. It can leach out from these materials during washing and sterilization processes or after landfilling. The endocrine disrupting effects of bisphenol A are exerted through binding and activating the estrogen receptor, a nuclear receptor. As a consequence, bisphenol A acts as a potential risk factor for the development of prostate and breast cancer and is suspected to reduce the number of sperm cells in men. Tributyltin has been widely used in antifouling paints and is found in high concentrations in the vicinity of shipping routes and in harbor sediments. Through binding and activation of the retinoid X receptor, also a nuclear receptor, tributyltin induces a phenomenon called “imposex” in female gastropods, which means that in addition to female sex organs a penis and *vas deferens* are formed.

The occurrence of both compounds, bisphenol A and tributyltin, can be monitored and quantified by the use of analytical methods such as HPLC, LC-MS and GC-MS. However, they are time-consuming, require high amounts of environmentally hazardous solvents and eluents as well as specific technical equipment and competence. In contrast, microbial bioassays offer inexpensive, easy-to-use and small-scale measurements. Furthermore, they can be employed to assess bioavailability. In this work, a receptor-based bioluminescent yeast assay was developed that has the advantage of qualitatively monitoring the endocrine disrupting effect of the compound as well as its metabolites. Furthermore, the lignin-decomposing abilities of litter-degrading fungi were exploited. These fungi produce highly active extracellular oxidative enzymes such as laccase and manganese peroxidase. These enzymes degrade recalcitrant substances such as plant lignin but also persistent environmental chemicals.

A novel bioluminescent yeast assay was constructed and characterized to detect organotin compounds such as tributyltin, triphenyltin and their metabolites. A chimeric human retinoic X receptor alpha is expressed from a plasmid. Upon binding of tributyltin or another ligand, expression of a luciferase reporter gene is triggered. After addition of D-luciferin, light is emitted and detected luminometrically. The assay has proven to be highly specific towards organotin compounds and natural ligands of the retinoic X receptor. Tributyltin was detected in nanomolar concentrations. At these low concentrations, tributyltin already exerts an endocrine disrupting effect in nature. Experiments were performed in small-scale and high-throughput manner and results of one analysis were obtained within one working day.

Next, a novel sampling method to determine extracellular fungal enzymes in agar was developed. Small pieces of growing solid-state fungal cultures were placed in a centrifugation tube filter, containing a cellulose acetate membrane. Centrifugation recovered water-soluble material including many enzymes. The recovery of two added model enzymes, laccase and manganese peroxidase, was in the range of 50 to 75 %. This method allowed the collection of spatial data from very small and defined areas of solid fungal cultures. It is also very well suitable for screening approaches, i.e. it can combine toxicity tests and the investigation of the influence of toxic/endocrine disrupting compounds on enzyme production of the fungus under study.

Finally, the degradation of the endocrine disrupting compound bisphenol A was studied in detail. Cultures of the litter-degrading fungi *Stropharia rugosoannulata* and *Stropharia coronilla* as well as a partially purified neutral manganese peroxidase from the latter were used to successfully degrade the estrogenic compound bisphenol A in culture medium or cell-free reaction solution, respectively. A bioluminescent yeast assay, expressing the estrogen receptor alpha, was employed to follow the removal of estrogenic activity. *S. coronilla* was shown to be the most efficient fungus; the estrogenic activity was reduced by 100 % in liquid cultures as well as during treatment with manganese peroxidase. In cultures of *S. rugosoannulata*, the estrogenic activity declined as well but temporarily re-appeared. Selected samples from this cultivation were additionally investigated for potential metabolites using LC-MS analysis and a pathway of bisphenol A conversion was hypothesized.

Tiivistelmä

Hormonihäiritsijöiksi kutsutaan yhdisteitä, jotka ovat hormonien kaltaisia, ja jotka voivat häiritä elimistön normaalia toimintaa. Hormonit toimivat elimistössä avain-lukko-periaatteella: hormoni on ”avain”, joka sitoutuu vastaanottavaan molekyyliin, reseptoriin eli ”lukkoon”. Hormonihäiritsijä bisfenoli A:ta käytetään polykarbonaattimuovien pehmittimenä, joita taas käytetään elintarvike-pakkauksissa, säilykepurkkien pinnoitteissa ja tuttipulloissa. Muoveja pestessä, steriloidessa tai kaatopaikkasijoituksessa bisfenoli A:ta voi huuhtoutua pesuveteen. Altistuminen bisfenoli A:lle voi aiheuttaa eturauhas- tai rintasyöpää ja sen on myös epäilty vähentävän siittiöiden määrää. Hormonihäiritsijä tributyylitina on ollut yleisesti käytössä veneenpohjamaalien tehoaineena, ja sitä on päätynyt suuria määriä laivaväylien läheisyyteen ja satamien pohjasedimentteihin. Maaleilla on pyritty estämään kotiloiden kiinnittyminen laivojen runkoon, sillä kotiloiden massa lisää laivan polttoaineenkulutusta. Tributyylitina aiheuttaa kotiloille, kuten merirokolle ja etanalle ”maskulinisaatiota”, ts. tributyylitinalle altistuneelle naaraskotilolle saattaa kehittyä penis tai muita uroskotiloiden elimiä.

Hormonihäiritsijöihin voidaan vaikuttaa kahdella tavalla: ongelmaa valvomalla, jolloin tilanteesta saadaan kokonaiskuva, ja poistamalla ongelma. Tässä työssä kehitettiin bioluminesenssiin perustuva hiivatesti, jossa geneettisesti muunneltu leivinihiiva tuottaa valosignaalin tributyylitinan läsnä ollessa. Testin avulla tributyylitina ja sen hormonaalisesti aktiiviset hajoamistuotteet voidaan havaita näytteestä nopeasti ja tarkasti. Lisäksi näytettä tarvitaan vain pieni määrä, ja tulokset saadaan yhden työpäivän aikana. Karikkeenlahottajasienten orgaanisten aineiden hajotuskykyä voidaan hyödyntää hormonihäiritsijöiden hajottamiseen eli ongelman poistamiseen. Luonnossa nämä sienet hajottavat kasvimateriaalia, kuten lehtiä ja oksia tuottamallaan solunulkoisilla, voimakkaasti hapettavilla entsyymeillä. Tässä työssä kehitetyn toisen menetelmän avulla voidaan helposti löytää sienilajeja, joilla on tehokas entsyymintuotto. Agarmaljalla kasvatetusta sienirihmasta erotetaan entsyymit yhdistetyn suodatuksen ja sentrifugoinnin avulla, ja entsyymiaktiivisuus määritetään suodoksesta. Valittuja sieniä voidaan hyödyntää prosessissa, jossa sieni tuottaa hapettavia entsyymeitä ja hajottaa hormonihäiritsijät.

Karikkeenlahottajasienet *Stropharia rugosoannulata* (viljelykaulussieni) ja *Stropharia coronilla* (nurmikaalussieni) valittiin bisfenoli A:n hajotuskokeeseen. Seurattaessa yhdisteen hajoamista hiivatestillä, havaittiin, että sekä *S. coronilla* sieni että sienen tuottama mangaaniperoksidaasi-entsyymi yksin pystyivät hajottamaan bisfenoli A:n täydellisesti. *S. rugosoannulata* sienen kasvatusliemessä bisfenoli A:n hormonaalinen aktiivisuus myös hävisi, mutta myöhemmin aktiivisuus palautui osittain. Kasvatusliemen analyysi paljasti, että vaikka suurin osa bisfenoli A:sta hajosi harmittomiksi yhdisteiksi, pieni osa muuntui bisfenoli A:n kaltaiseksi hormonihäiritsijäksi.

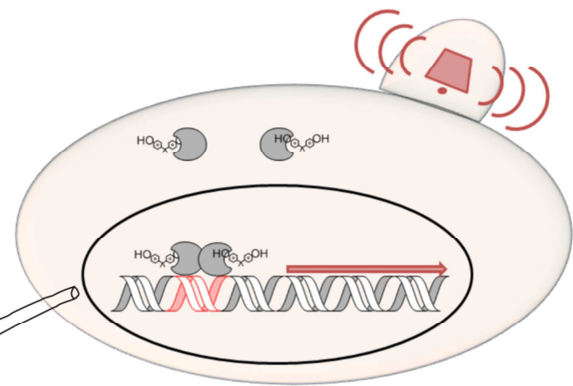
Työssä kehitettyjen menetelmien avulla hormonihäiritsijöiden hajotus on mahdollista ja hajoamisen seuraaminen on helppoa. Seurantatestin avulla selviää myös milloin tarkentavat kemialliset analyysit ovat tarpeen.



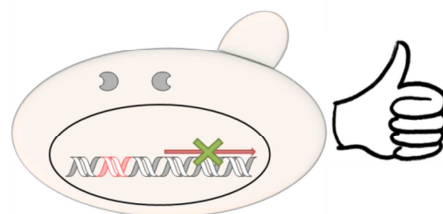
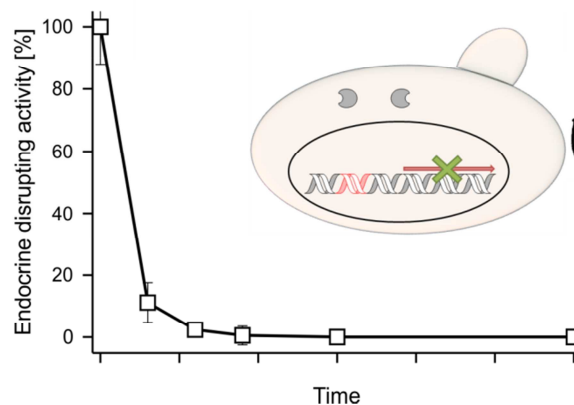
Pollutant-degrading fungus



**Successful
degradation
of endocrine
disrupting
compounds**



**Yeast reporter
to monitor the
endocrine
disrupting effect**



Abbreviations

AFS Convention	Convention on the Control of Harmful Anti-fouling Systems on Ships
BPA	Bisphenol A
CYP	Cytochrome P450 monooxygenase system
DBT	Dibutyltin
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
EDC	Endocrine disrupting compound
ER α	Estrogen receptor α
ER β	Estrogen receptor β
ERE	Estrogen response element
GC-MS	Gas chromatography-mass spectrometry
HBT	1-hydroxybenzotriazole
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
<i>luc</i>	<i>Photinus pyralis</i> (firefly) luciferase gene
MBT	Monobutyltin
MnP	Manganese peroxidase
MW	Molecular weight
OD	Optical density
PEG	Polyethylene glycol
QSAR	Quantitative structure activity relationship
RXR	Retinoid X receptor
TBT	Tributyltin
UPO	unspecific peroxygenase
VDSI	Vas Deference Sequence Index

List of original publications

I Grit Kabiersch, Johanna Rajasärkkä, Marja Tuomela, Annele Hatakka, Marko Virta and Kari Steffen (2013) Bioluminescent yeast assay for detection of organotin compounds. *Analytical Chemistry* dx.doi.org/10.1021/ac4003062

II Jussi Heinonsalo, Grit Kabiersch, R. Maarit Niemi, Suvi Simpanen, Hannu Ilvesniemi, Martin Hofrichter, Annele Hatakka, Kari Steffen (2012) Filter centrifugation as a sampling method for miniaturization of extracellular fungal enzyme activity measurements in solid media. *Fungal Ecology* 5 (2): 261-269

III Grit Kabiersch, Johanna Rajasärkkä, René Ullrich, Marja Tuomela, Martin Hofrichter, Marko Virta, Annele Hatakka, Kari Steffen (2011) Fate of bisphenol A during treatment with the litter-decomposing fungi *Stropharia rugosoannulata* and *Stropharia coronilla*. *Chemosphere* 83 (3): 226-232

The Author's Contribution

I Grit Kabiersch planned and conducted the experiments, analyzed and interpreted the results, wrote the paper and is the corresponding author. Johanna Rajasärkkä constructed the yeast and participated in discussion and writing of the manuscript.

II Grit Kabiersch was involved in planning and conducting the culture test experiments, analyzed and interpreted the corresponding results, wrote the paper together with Jussi Heinonsalo and is the corresponding author.

III Grit Kabiersch planned and conducted the experiments, analyzed and interpreted the results, wrote the paper and is the corresponding author.

1 Silent Spring, Underkastelsen, Our Stolen Future

“In the gutters under the eaves and between the shingles of the roofs, a white granular powder still showed a few patches; some weeks before it had fallen like snow upon the roofs and the lawns, the fields and streams.” (A Fable for Tomorrow, Carson 2002)

In 1962, Rachel Carson published her famous book “Silent Spring”. In her introductory chapter, “A Fable for Tomorrow”, she describes a dramatic scenery: farmers and children are struck by sudden mysterious illnesses, some of them die, hens breed but their chicken do not hatch, litters of farm animals are born dead, trees bloom but there are no insects to pollinate them, there are no fish in the rivers and all birds have disappeared – silent spring has arrived. The following chapters list health hazards due to uncontrolled large-scale spraying of arable land with poisonous insecticides but also focus on economic losses. For example, non-selective killing of insects led to a decrease of young salmon, which are feeding on stream insects. In the 1950’s, this reckless practice resulted in the break-down of the commercial and recreational fishing industry in the US (Rivers of Death). With “her remarkable knack for taking dull scientific facts and translating them into poetical and lyrical prose that enchanted the lay public” (Leonard 1997) she reached a broad audience and started an environmental movement (Koch-Kanz & Pusch 2005). One of Rachel Carson’s biggest achievements was the ban of the domestic production of the pesticide **dichlorodiphenyltrichloroethane** (DDT) and bringing the (mis)use of chemical pesticides and other treatments to public attention (Introduction to Silent Spring, Lear 2002).

Today, 50 years later, the widespread use of chemicals is strictly regulated in developed countries. Nevertheless, an increasing number of chemicals are released into the environment, unleashing new problems. These new challenges prompted Swedish film director Stefan Jarl to publish the documentary “Underkastelsen – Submission” about modern people’s chemical burden (Jarl 2010). As a kind of self-experiment, he donated some blood in order to get it analyzed for man-made chemicals. Throughout the film, Jarl was accompanied by professor Åke Bergman from Stockholm University, who, besides analyzing his blood, explained the source and relevance of the chemicals. Bergman found a chemical cocktail of several hundred single substances. In addition, Stefan Jarl interviewed many experts including Fredrick vom Saal (University of Missouri), Andreas Kortenkamp (University of London), Ana M. Soto (Tufts University), among others. During the film, topics slightly changed from knowledge about animal experiments to well-known effects on humans such as the development of cancer, and from general toxicity to effects on the reproductive system. One’s own fertility is an extremely vulnerable point for almost everyone. An even more vulnerable point was hit when Stefan Jarl convinced the 35 years younger actress Eva Röse to test her blood as well. She was pregnant and gave birth while the film was made. Her chemical burden was not as high as Jarl’s (yet), but she learned that when her baby is born, man-made chemicals will already be running in its blood stream and even more will gather during breast feeding.

Although society is aware and sensitized, a huge amount of chemicals are still being released into the environment and accumulate in human bodies. Today, people do not face the problem of acute toxicity, but long-term, low-dose and chronic effects. Furthermore, in recent years, attention has been drawn to the interference of man-made chemicals with the endocrine system. The term “endocrine disruptor” to describe an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour was coined in 1991, during a meeting of 22 scientists at Wingspread, Racine, Wisconsin, USA (Vandenberg *et al.* 2009). One more consequence of this meeting was the publication of another popular scientific book, “Our Stolen Future” by Theo Colborn, Dianne Dumanoski, and John Peterson Myers (Colborn *et al.* 1997). This book describes, in many examples, that, as already mentioned, the threat through endocrine disrupting compounds is not immediately obvious, but recognized only after many years and, very often, for the first time in our children.

These examples demonstrate that ubiquitous and permanent exposure to endocrine disrupting compounds is an issue of high concern. In the following Chapter 2, I will provide a literature review about the use, harmful effects and legislation of the endocrine disrupting compounds bisphenol A and tributyltin. A great amount of research has been done to describe and understand endocrine disruption. As a consequence, production and application of these substances is banned or regulated more strictly. They will, nevertheless, pose problems for years. As another consequence, attempts are going on to restore contaminated environment. However, there is no patent remedy yet, in which a specialist simply opens a toolbox and starts his work. One reason for this is that the variety of chemicals released into the environment also requires a variety of tools. The fungal toolbox described in this work (Chapter 5) is based on biological methods, which are employed for monitoring as well as degradation of endocrine disrupting compounds. The same chapter will provide a theoretical background of the tools (Paragraphs 5.1.1, 5.1.2, 5.2.1), followed by the achievements leading to **Publications I-III** (summarized in Paragraphs 5.1.3, 5.2.2, 5.3) and a strategy for the application of the tools (Paragraph 5.4).

2 Endocrine disruption

2.1 Endocrine disrupting compounds

Endocrine disrupting compounds (EDCs) are synthetic or natural compounds that mimic the action of natural hormones and thus disrupt or alter functions of the endocrine system usually through direct interactions with nuclear receptors (Inoue *et al.* 2011). Normally, the hormone binds the target receptor in a “lock and key” mechanism. This complex moves into the nucleus, activates target genes and produces a biological response (Figure 1A, Colborn *et al.* 1997). Substances that mimic a hormone bind to the same target receptor and induce a response in the absence of the actual hormone (Figure 1B). Furthermore, there are substances that act as hormone blockers. They prevent natural hormones from interacting with the receptor but they do not induce a response (Figure 1C). Additionally, the concentration and availability of natural hormones is regulated by, for example, steroid-binding plasma proteins (Anderson 1974). Steroid-binding plasma proteins regulate the level of free and consequently biologically active hormones. However, they do not bind artificial hormonally active compounds (Anderson 1974). As a result, small concentrations of an endocrine disruptor or a compound with lower receptor affinity, a so-called “weak” EDC, might still have a large effect (Figure 1D).

Compared to classic toxins, where the dose makes the poison and the effects are evident shortly after exposure, consequences of endocrine disruption may be rather subtle but they are not less dramatic. EDCs are effective at very small concentrations; they can pass the placenta and cause long-term effects. It is important to note that the severity of the effect depends on the timing of exposure.

Vom Saal *et al.* (1999) demonstrated in a series of experiments with mice that already small, naturally occurring differences of hormone levels during embryonic development can create enormous differences in the behavior of siblings. They observed one aggressive female mouse for every six female mice. In their study, they marked pups according to their position in the uterus just before they were born and drew a clear relationship between intrauterine position and aggressiveness. Aggressive females were the ones who had been sandwiched between males. This intrauterine position phenomenon, resulting in different behavior of siblings, was caused by altered levels of testosterone and estradiol in parts per trillion and parts per billion and is a classic example of the sensitivity and vulnerability of the hormone system (Vom Saal *et al.* 1999).

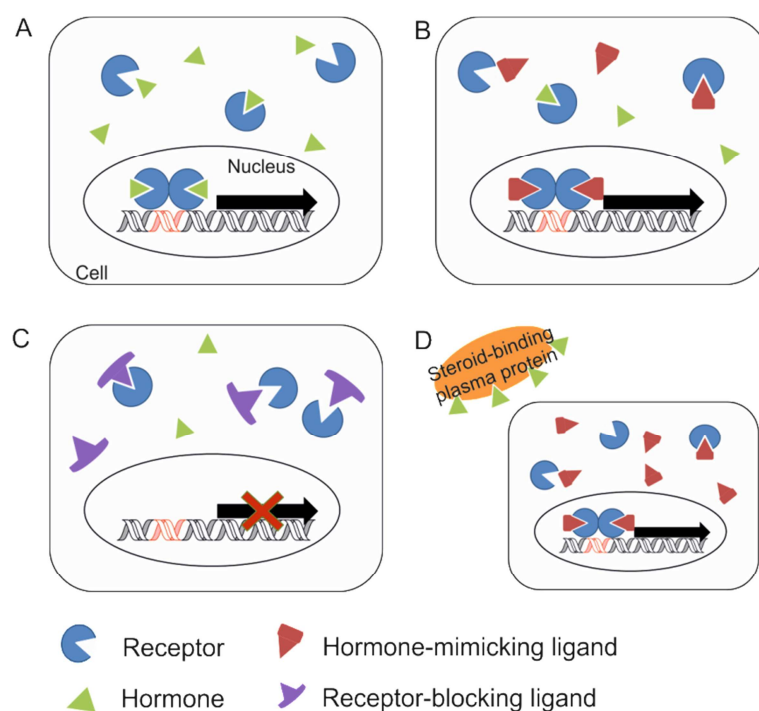


Figure 1: Mechanisms of endocrine disruption. (A) Normal process: natural hormones bind to the target receptor and induce a response. (B) Hormone-mimicking ligands compete with natural hormones for receptor binding and induce a response. (C) Receptor-blocking ligands prevent natural hormones from binding to the target receptor and from inducing a response. (D) Steroid-binding plasma proteins bind natural hormones. Hormone-mimicking ligands bind to the receptor and induce a response. (Adapted from Colborn *et al.* 1997)

The two pharmaceutical compounds diethylstilbestrol (DES) and thalidomide (Contergan) demonstrate the dramatic consequences of interference with the hormone system. Thalidomide was used in the 1950s to treat insomnia and nausea during early pregnancy. Especially when taken during early pregnancy, the drug caused malformation or absence of limbs and an uncounted number of stillbirths (Teo 2005). A metabolite of thalidomide, formed in the liver, is supposed to inhibit angiogenesis, the formation of blood vessels in the developing fetal limb bud through modulation of the transmembrane receptors integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (D'Amato *et al.* 1994, Kenyon *et al.* 1997, Kruse *et al.* 1998). The thalidomide scandal certainly contributed to the complete destruction of the myth that the placental barrier protects the unborn. Also DES is a substance able to cross the placenta but, in contrast to thalidomide, the effects became obvious only decades later. DES was used to prevent miscarriages from the 1940s on. However, it took many years until scientists and physicians established a connection between exposure to the drug during early development and the occurrence of rare vaginal cancer or abnormally formed uteri in girls as well as abnormal sperm, undescended testicles or reduced fertility in men are related to the exposure of DES during early development (Harris & Waring 2012, Laronda *et al.* 2012). DES is a high-affinity agonist of the estrogen receptors ER α and ER β (Kuiper *et al.* 1997) and is

commonly used as a reference compound to investigate hormonally-induced cancers (Davis *et al.* 2012). Furthermore, DES can activate an ER-mediated signalling pathways that results in the epigenetic alterations of genes. It is well known that hypermethylated genes are frequently found in breast cancer tissue (Doherty *et al.* 2010, Hsu *et al.* 2009).

2.2 Bisphenol A

Bisphenol A (BPA) is an endocrine disrupting compound with functional phenolic groups linked through a propane moiety (Figure 2A). BPA is used for the manufacture of polycarbonate plastics (Figure 2B) and epoxy resins (Figure 2C). The ester bonds in polycarbonate plastics are subject to hydrolysis resulting in leaching of BPA into the environment (vom Saal & Hughes 2005). Warnings have also been issued about leaching from epoxy resins. These polymers are, for example, used to reline water pipes (International Chemical Secretariat ChemSec).

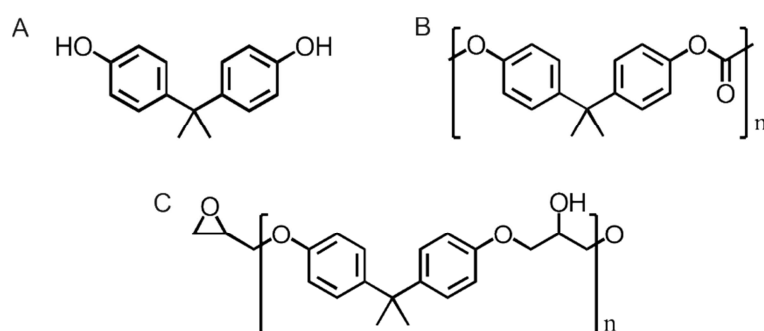


Figure 2: (A) Bisphenol A, (B) Structure of polycarbonate plastics, (C) Structure of bisphenol-A diglycidyl ether epoxy resins.

The estrogenic activity of BPA was first described in 1936 (Dodds & Lawson 1936) but the consequences of these early findings were demonstrated in a publication by Krishnan *et al.* only in 1993. Surprisingly, an estrogenic substance was found in the cultivation medium of *Saccharomyces cerevisiae* (Krishnan *et al.* 1993). This substance was able to compete with [³H] estradiol for the binding site of estrogen receptors in rat uterus cells and was finally identified as bisphenol A by nuclear magnetic resonance and mass spectroscopy. Furthermore it was demonstrated that BPA was not produced by the yeast but that it originated from the distilled water used for the preparation of the yeast medium. However, BPA was only found in distilled water that had been autoclaved in polycarbonate flasks (Krishnan *et al.* 1993). This laboratory detective story that began with the investigation of the surprising occurrence of an estrogenic substance in yeast cultures impressively demonstrates that BPA leaches from polycarbonate plastics and that it may disturb more than the outcome of experiments.

BPA exerts its endocrine disrupting effect through binding and activation of estrogen receptors (Kuiper *et al.* 1998, Welshons *et al.* 2006). The ligand-binding domain of the estrogen receptor has a cavity size of 450 Å³ (Anstead *et al.* 1997). It forms a pincer-like arrangement around the A-ring of the natural ligand 17β-estradiol (molecular volume of 245 Å³); the remaining space of the ligand-binding pocket is very flexible and can accommodate a number of different hydrophobic groups (Figure 3A, Brzozowski *et al.* 1997). Receptor-binding studies as well as quantitative structure activity relationship (QSAR) modeling revealed that estrogen receptors also accept non-steroidal ligands with phenolic groups in *para* orientation such as BPA (Figure 3B, Coleman *et al.* 2003, Kuiper *et al.* 1998). Nevertheless, the affinity of BPA for estrogen receptors is 10,000 to 100,000-fold lower (Vandenberg *et al.* 2007).

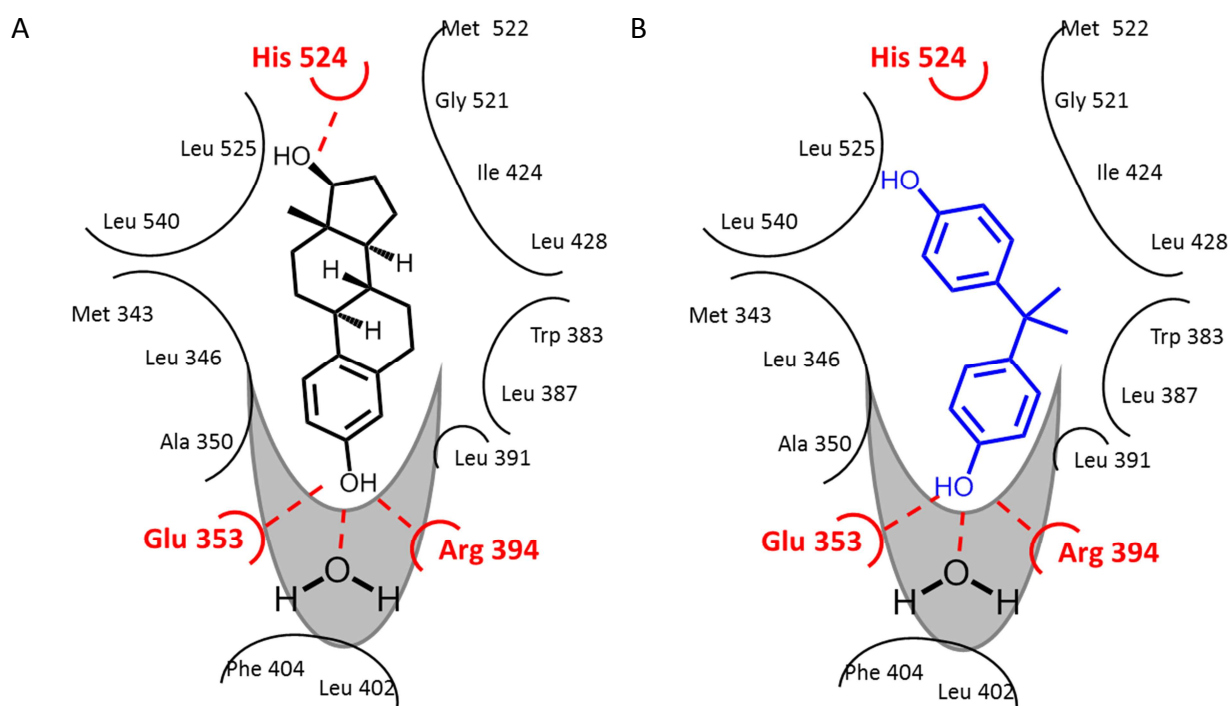


Figure 3: Schematic representation of the estrogen receptor ligand-binding domain accommodating 17β estradiol (A) or bisphenol A (B). Important interactions with amino acids are highlighted in red. The “pincer-like arrangement” around the phenolic moiety is illustrated as a grey shape (adapted from Brzozowski *et al.* 1997).

Estrogen receptors are found in numerous tissues of both males and females. Thus, abnormal presence of estrogenic compounds in these tissues may cause a large spectrum of negative effects (Janošek *et al.* 2006). For BPA, changes in cell functions have been observed at extremely low concentrations, starting from 1 pM (Segner *et al.* 2003, vom Saal & Hughes 2005, Welshons *et al.* 2006). As examples, it reduces fertilization success in zebrafish (*Danio*

rerio) in the ng/l-range (Segner *et al.* 2003) or stimulates the production of additional female organs (superfeminization), which ultimately result in the rupture of the oviduct and increased mortality in the giant ramshorn snail (*Marisa cornuarietis*) at concentrations starting from 1 µg/l (Oehlmann *et al.* 2000, Weltje *et al.* 2005). Altered population size in turn affects ecosystem functioning as well as food chains and thus, the effects of BPA have eventually reached humans as the top predators (Oehlmann *et al.* 2007). Acting on humans directly, BPA is suspected to reduce sperm production in men and to increase the risk of breast and prostate cancer (Maffini *et al.* 2006).

In 2006, the worldwide annual production of BPA was 3.8 million tons (Umweltbundesamt 2010). Humans mainly get into contact with BPA through food sources. BPA has been found in various food packaging materials and paper table cloths (Pérez-Palacios *et al.* 2012) from where it may leach into the food and get ingested. In a study designed by Carwile *et al.* (2011), volunteers consumed canned soup or a soup prepared from fresh non-canned ingredients. As a result, BPA was detected in the urine of 100 % of those volunteers, who had consumed canned soup (vs. 77 % for the other group) with mean concentrations of 20.8 µl/l (vs. 1.1 µl/l). Another every-day product containing BPA are shopping receipts made of thermal printing paper. Biedermann *et al.* (2010) found out that touching the paper with dry hands resulted in a transfer of, on average, 1 µg BPA to the finger pad. Touching the paper with humid or greasy hands resulted in a transfer of up to 23 µg BPA per finger pad. It is not clear whether BPA can permeate skin (Biedermann *et al.* 2010), however, from the fingers BPA will easily find its way into the mouth and from there into the body. Consequently, BPA has been found in body fluids such as urine and breast milk (Carwile *et al.* 2011, Mendonca *et al.* 2012).

Official governmental publications state that exposure to BPA at current environmental levels does not represent a risk factor for human health (Umweltbundesamt 2010). However, vom Saal and Hughes (2005) as well as Welshons *et al.* (2006) published extensive literature reviews about low-dose effects of BPA and pointed out that BPA exposure can be especially harmful during vulnerable periods of development. As a consequence, Canada prohibited advertisement, sale and import of polycarbonate baby bottles containing BPA, from 11th of March 2010 on (Canada Gazette 2010). In the EU, manufacture of these bottles is prohibited since 1st of March 2011; sale and import is prohibited since 1st of June 2011 (Barroso 2011). Other countries such as Malaysia, South Africa and China followed suit (Sustainability Support Services Europe).

The major source of BPA contamination in the environment is leaching from landfills (Kamata *et al.* 2011, Yamamoto *et al.* 2001) and wastewaters. As an example, water samples were taken from areas, in which paper-recycling process water had been discharged, and BPA was detected at two of the five tested sites in the Shizuoka Prefecture in Japan (Terasaki *et al.* 2007). Generally, degradation rates for BPA in sewage treatment plants vary greatly for different plants and treatment technologies. However, Ying *et al.* (2008) found a removal efficiency of around 90 % in most of the investigated plants. Especially oxidation processes

and treatment with activated sludge had a positive effect on the removal efficiency. Heemken *et al.* (2001) investigated the occurrence of bisphenol A, alkylphenols and alkylphenol ethoxylates in water and sediment of the river Elbe in Germany, from the Czech border to the North Sea estuary. Increases in BPA concentrations were found at sampling points downstream of a BPA producing factory and downstream of the city of Dresden. BPA concentrations in river water samples were usually within the three-digit ng/l-range and one order of magnitude lower in marine environment (Arditsoglou & Voutsas 2012, Belfroid *et al.* 2002, Heemken *et al.* 2001, Ying *et al.* 2008). Furthermore, BPA was found in fish at locations where no BPA had been detected in the water (Belfroid *et al.* 2002).

2.3 Tributyltin

Tributyltin (TBT) is one of the most toxic organotin compounds. Organotin compounds represented as RSnX_3 , R_2SnX_2 , R_3SnX or R_4Sn are organic derivatives of a tin atom (Sn^{4+}), covalently bound to one or more alkyl or aryl groups (R) and anionic species (X) such as halide, oxide or hydroxide (Hoch 2001, Okoro *et al.* 2011). Inorganic tin is considered to be non-toxic, whereas tri-substituted organotin compounds such as TBT exhibit highly toxic characteristics. In nature, tributyltin is degraded into dibutyltin (DBT) and monobutyltin (MBT) via successive dealkylation (Figure 4). The reduction of butyltin chains is accompanied with a reduction of general toxicity (Hoch 2001, Okoro *et al.* 2011). However, each of these dealkylation steps has a half-life of roughly two years in sediments (Sarradin *et al.* 1995).

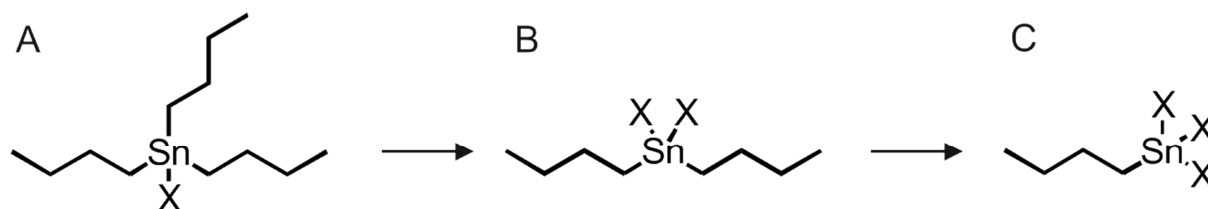


Figure 4: Degradation of tributyltin (A) to dibutyltin (B) and monobutyltin (C). X = halide, oxide or hydroxide.

In recent years, TBT has been in the center of public and academic interest for several reasons: (1) in 2003, the global production was at about 40,000 t (Okoro *et al.* 2011); (2) its application as a biocide comprises usage in antifouling paints on ships, for wood preservation, in industrial water systems such as cooling towers and refrigeration water systems, or as a fungicide on textiles (Antizar-Ladislao 2008); (3) it has low solubility in water (50 mg/cm^3) and adheres to sediment (Hoch 2001); (4) adsorption to sediments is reversible and thus provides a long-term source of contamination of the overlying water column and for filter- and sediment-feeding organisms (Antizar-Ladislao 2008, Okoro *et al.* 2011); (5) it is widely distributed around the world (Tittley-O'Neal *et al.* 2011); and (6) it causes imposex in

female gastropods (Gibbs & Bryan 1986, Gibbs *et al.* 1990, Nishikawa *et al.* 2004, Stange *et al.* 2012).

Imposex is a phenomenon observed in female gastropods. In addition to the female genital system, male organs such as penis and/or vas deferens are formed (Okoro *et al.* 2011). This phenomenon occurs literally everywhere in the world, from remote areas such as Antarctic waters (Negri & Marshall 2009) to well-frequented spots such as harbors (Cao *et al.* 2009, Castro *et al.* 2012, Oliveira *et al.* 2009). Because of its ubiquity, imposex is used as a biomarker for organotin contamination. Usually, these contamination studies report the percentage occurrence of imposex, mean values of penis length and the Vas Deference Sequence Index (VDSI), which quantifies the degree of vas deferens development in imposex-inhabiting females (Gibbs *et al.* 1987, Nishikawa *et al.* 2004, Titley-O'Neal *et al.* 2011). The most-affected gastropods belong to the Muricidae (rock snails), Buccinidae (true whelks), Nassariidae (dog whelks) and Conidae (cone snails) families. The most-studied species are the dog whelk *Nucella lapillus*, the netted dog whelk *Nassarius reticulatus*, *Thais clavigera* and the common periwinkle *Littorina littorea* (Titley-O'Neal *et al.* 2011). Nishikawa *et al.* (2004) demonstrated in the rock shell, *Thais clavigera*, that substantial penis growth and development of the vas deferens was, in fact, induced by organotins and that tributyltin and triphenyltin exert their endocrine disrupting effect via binding to retinoid X receptors.

Retinoid X receptors (RXRs) are nuclear receptors, which are expressed in all tissues of the human body. The RXR α subtype is mainly expressed in liver, lung, muscle, kidney, epidermis and intestine, RXR β is expressed ubiquitously and RXR γ localizes to brain, cardiac and skeletal muscles (Pérez *et al.* 2012). RXRs can form homodimers upon ligand binding and heterodimers with a range of receptors including retinoic acid receptors, thyroid hormone receptors, vitamin D receptors, proliferator-activated receptors, liver X receptors and farnesoid X receptors (Kliwer *et al.* 1992, Le Maire *et al.* 2009, Pérez *et al.* 2012, Yu *et al.* 1991, Zhang *et al.* 1992). Thus, they can act as transcription factors in many signaling pathways and interference may result in a broad spectrum of consequences.

RXRs have an L-shaped ligand-binding pocket, which is formed by helices H3, H5, H7, H11 and the β -turn. It has a volume of 489 Å³ (Bourguet *et al.* 1995, Egea *et al.* 2000). The natural ligand, 9-*cis* retinoic acid (291 Å³), occupies 59 % of the cavity and forms 77 contacts with amino acid residues, i.e. R316 seals the elongated pocket and interacts with the carboxylate group of the retinoic acid, F313 interacts with the isoprenic carbons C11-C14, and, on the other side of the pocket, C432 interacts with the C6 β -ionone ring carbon and the isoprenic C7 carbon (Figure 5A, Egea *et al.* 2000). The hinge region bears unoccupied volume and seems to be the most flexible region (Egea *et al.* 2002). For this reason, RXRs can bind other compounds such as polyunsaturated fatty acids (Egea *et al.* 2002) or TBT, which is suggested to form a covalent Sn-S bond with C432 (Figure 5B, Le Maire *et al.* 2009).

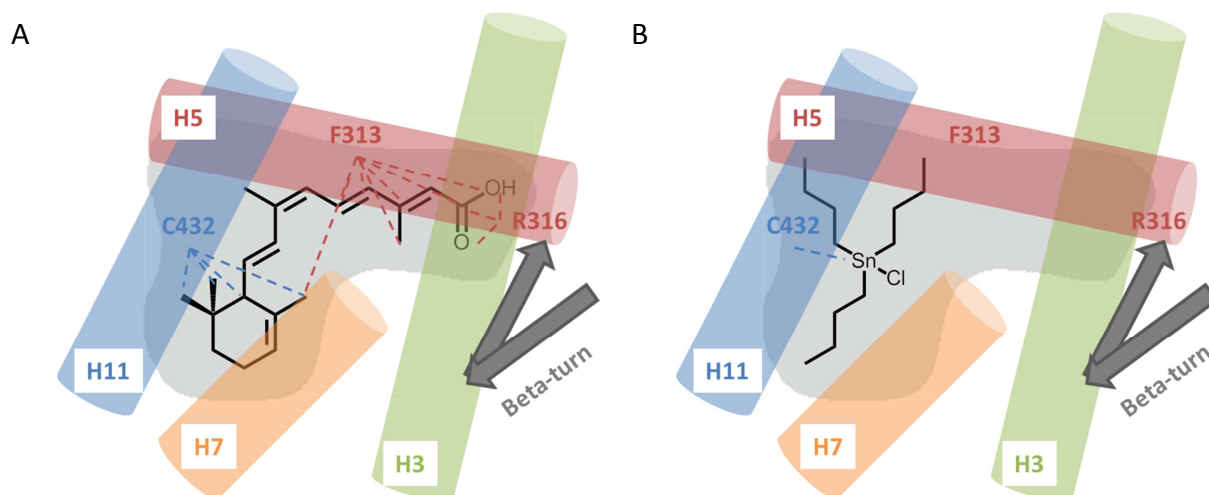


Figure 5: Schematic drawing of the retinoid X receptor ligand-binding pocket accommodating 9-*cis* retinoic acid (A) and tributyltin (B). Helices are drawn as barrels, beta-turns are presented as arrows. Important interactions with amino acids are highlighted. The L-shaped binding pocket is illustrated as a grey shape. (Adapted from Egea *et al.* 2000, Le Maire *et al.* 2009)

The main sources of environmental pollution with TBT are antifouling paints on ship hulls. Fouling, the attachment of marine organisms to ship hulls, reduces speed and consequently increases fuel consumption, which is an economical but also an environmental problem. Antifouling paints counteract this disadvantage. Furthermore, they prevent the introduction of invasive alien species to already established ecosystems. Over time, TBT is released from the paint, forms a thin layer of concentrated TBT around the hull and kills or prevents the attachment of organisms such as barnacles. However, TBT diffuses further and harms also non-target organisms. It accumulates and biomagnifies through the food chain, in which humans are the top predators (Okoro *et al.* 2011). In the late 1970s, it was noticed that TBT induced shell malformations in Pacific oysters at the Atlantic coast (Table 1, Huggett *et al.* 1992). In 1982, France banned the use of TBT-containing antifouling paints for boats shorter than 25 m. National regulations followed in 1985 in the UK, in 1988 in the USA and in 1990 in Japan. Additionally, conventions were held to protect larger areas such as the Mediterranean or the Baltic Sea (Table 1, Gipperth 2009, Huggett *et al.* 1992, Sonak *et al.* 2009). Furthermore, a global regulation of the use of TBT-based antifouling paints was discussed and, in 1998, an international ban of TBT was proposed. The Convention on the Control of Harmful Anti-Fouling Systems on Ships (AFS Convention) banned the use of all organotin compounds in antifouling paints with effective dates of 1st January 2003 and 1st January 2008. From 1st January 2003, organotin compounds must not be applied or re-applied to ships. From 1st January 2008 organotin compounds must not be found on the hull or external parts or surfaces of ships. However, Article 18 states that the AFS Convention will enter into force only 12 months after 25 states representing at least 25 % of the world's merchant shipping tonnage have ratified it. Panama signed the AFS Convention as the 25th

member state, which now represents 38.1 % of the world's shipping tonnage; the second effective date was 17th September 2008 (Senda 2009). Also the EU agreed on prohibiting the use of organotin compounds with effective dates of 1st July 2003 and 1st January 2008 (Table 1, Gipperth 2009). Both the AFS Convention and the EU regulation include the clause that the prohibition is binding for ships, which flag under the member states as well as for ships that enter a member state port (Gipperth 2009, Sonak *et al.* 2009). Hence, ships not operating under EU and AFS Convention member states' flags and using none of the member states' ports may still use TBT-based antifouling paints. Thus, the risk of TBT contamination has not ceased to exist for regional trades between non-member states, especially in and between developing countries with fast growing economies such as India, and for the repair and ship breaking industry in less regulated countries (Sonak *et al.* 2009). Furthermore, TBT is still in use as a biocide in agriculture. Phasing out TBT will generate large amounts of hazardous waste, emerging from abrasive materials and hydroblasting waste water. Last but not least, TBT has very low degradation rates in sediment and thus, poses the risk of permanent remobilization and recontamination of the overlying water column (Dubey & Roy 2003, Kotrikla 2009).

Table 1: Timeline for the ban of TBT (Gipperth 2009, Huggett *et al.* 1992, Senda 2009, Sonak *et al.* 2009)

Date	Steps to outphase TBT
Late 1970s	Occurrence of shell malformations in Pacific oysters at Arcachon Bay, Atlantic coast, France
1982	TBT-containing antifouling paints banned for boats shorter than 25 m in France
1985	Regulations to prevent the retail sales of TBT paints in the UK
1988	Restrictions for the use of TBT by “Organotin Antifouling Paint Control Act of 1988” in the USA: prohibition on retail sales of TBT antifouling paint additives and, application of TBT-containing paints on vessels shorter than 25 m
1990	Comprehensive restrictions on the use of TBT paint in Japan
1990	Active discussion of the need for a global regulation by the Marine Environment Protection Committee (MEPC)
1991	Barcelona Convention bans organotin-based antifouling paints on ships hulls shorter than 25 m in the Mediterranean
1992	Helsinki Convention bans the use of organotin compounds in antifouling paints for fishnet cages and pleasure crafts shorter than 25 m in the Baltic Sea
1998	Ocean Policy bans TBT on ships from January 2006 in Australia
1998	Basel Convention bans the export of ships coated with TBT paints
1998	Belgium, Denmark, France, Germany, Norway, the Netherlands, Sweden, the UK, and Japan propose an international ban of TBT
2001	Use of all organotin compounds in antifouling paints banned by the AFS Convention with effective dates of 1 st January 2003 and 1 st January 2008
1 st January 2003	Organotin compounds cannot be applied or re-applied to ships
17 th September 2007	Panama joins the AFS Convention as 25 th member state, which now represents 38.1 % of the world’s shipping tonnage
17 th September 2008	Organotin compounds should not be found on the hull or external parts or surfaces of ships
2003	EU regulation for the prohibition of the use of organotin compounds on ships with effective dates of 1 st July 2003 and 1 st January 2008

3 Objectives of this thesis

Humans are and will be permanently and continuously exposed to a wide variety of chemicals all over the world. Endocrine disrupting compounds as well as other harmful substances might be regulated more strictly or even banned, but many of them still pose a problem for years or might be even replaced by novel compounds with not yet known harmful effects. Among others, there are two major problems, which need to be solved:

1. Detection of the compounds: It is possible to detect very tiny amounts of known substances using analytical methods. However, this often requires extraction and derivatization methods and thus, the use of even more harmful chemicals. Furthermore, analytical description of contaminants alone does not predict the effect and harm on the environment. Thus, risk assessment often needs to be accompanied with animal tests.
2. Persistence of the compounds: Humans are permanently and continuously exposed to a wide variety of chemicals with known, partly known or not yet known harmful effects. Although production of harmful substances may be regulated and banned, many of them are persistent to natural degradation and thus, pose a problem for decades.

These two problems led to the following question:

Is it possible

a) to monitor

and

b) to degrade

endocrine disrupting compounds with biological methods and are they applicable?

The objective of this thesis is, with the help of a fungal toolbox, to, on the one hand, degrade endocrine disrupting compounds (EDCs) and, on the other hand, to prove that the endocrine disrupting effect can be completely removed. This thesis focusses on two EDCs, the estrogenic substance bisphenol A (BPA) and the virilizing substance tributyltin (TBT). Both substances interact with nuclear receptors, eliciting a biological response.

As a first tool, a bioluminescent yeast assay, displaying the particular target receptor, is used to detect the parent compounds and possible harmful degradation products through a light signal. The assay can be used to monitor endocrine disrupting activity qualitatively, rapidly and in small scale, to reduce the amount of harmful chemicals for analysis and to provide a rough prediction of potential biological effects.

The second tool is provided by litter-degrading fungi that produce highly active extracellular oxidative enzymes. These enzymes are not only capable of degrading recalcitrant plant lignin, but they can also break down a variety of persistent man-made chemicals. With these fungi and their enzymes it is possible to decompose EDCs. Because only relatively few fungi have been thoroughly studied for their degradation potential of EDCs, the aim of this doctoral work was to develop new methods to find novel fungi for efficient degradation of EDCs in the environment.

The objectives of this thesis are

- 1) The development and use of bioluminescent yeast assays to monitor endocrine disrupting activity in a small-scale and high-throughput manner. (**Publication I**)
- 2) The development of a rapid method in order to identify the most efficient enzyme-producing litter-degrading fungi in a single experimental set-up. (**Publication II**)
- 3) To study the biodegradation and fate of bisphenol A by two litter-decomposing fungi as degradation tool, a bioluminescent yeast assay as monitoring tool, subsequent chemical analysis of selected samples and development of a hypothetical pathway for the conversion of bisphenol A. (**Publication III**)
- 4) The development of a flexible strategy for the identification of efficient degraders and rapid upscaling for bioremediation attempts in pilot and large scale experiments.

4 Material and methods

4.1 Fungi

The fungal toolbox is composed of monitoring tools employing bioluminescent yeasts and degradation tools employing litter-decomposing fungi (Table 2). In addition to these fungi, several other fungi, including litter-decomposing, white-rot, brown-rot, ectomycorrhizal and root-endophytic fungi were used to develop a novel sampling method to screen for extracellular enzyme activities in solid agar media (**Publication II**) and they are listed as well in Table 2.

Table 2: List of fungal strains mentioned in Publication I-III and in the text. All strains are deposited at the University of Helsinki, Finland. FBCC refers to the culture collection number of the Fungal Biotechnology Culture Collection, the abbreviation in brackets refers to identification codes used before these strains were included into the FBCC.

Yeast strains used as monitoring tools	Used in
<i>Saccharomyces cerevisiae</i> BMA64/luc	I, III
<i>Saccharomyces cerevisiae</i> BMAEReluc/hRXR-ER	I
<i>Saccharomyces cerevisiae</i> BMAEReluc/ER α	III
Litter-decomposing fungi used as degradation tools	
<i>Stropharia coronilla</i> FBCC 480 (TM47-1)	III
<i>Stropharia rugosoannulata</i> FBCC 482 (11372)	II, III
<i>Agrocybe praecox</i> FBCC 476 (TM70.84)	II, 5.4
Fungi tested for extracellular enzyme activity	
<i>Cadophora finlandica</i> FBCC 1393 (JH163)	II
<i>Cenococcum geophilum</i> FBCC 1388 (JH101)	II
<i>Collybia dryophila</i> FBCC 626 (K209)	II
<i>Irpex lacteus</i> FBCC 384 (CCB-196)	II
<i>Hypholoma fasciculare</i> FBCC 1034 (CCBAS 281)	II
<i>Lactarius rufus</i> FBCC 1389 (JH84)	II
<i>Meliniomyces variabilis</i> FBCC 1394 (JH31)	II
<i>Ophiostoma abietinum</i> FBCC 1395 (JH142)	II
<i>Phanerochaete velutina</i> FBCC 941 (T244i)	II
<i>Phialocephala fortinii</i> FBCC 1396 (JH103)	II
<i>Physisporinus rivulosus</i> FBCC 397 (T241i)	II
<i>Piloderma olivaceum</i> FBCC1391 (JH33)	II
<i>Piptoporus betulinus</i> FBCC 703 (T35)	II
<i>Postia caesia</i> FBCC 757 (T91)	II
<i>Rhizoscyphus ericae</i> aggr. FBCC 1397 (JH135)	II
<i>Serpula lacrymans</i> FBCC 1009 (Md1144/VTT)	II
<i>Sphaerobolus stellatus</i> FBCC 253 (PO203)	II
<i>Stropharia aeruginosa</i> FBCC 521 (K47)	II
<i>Stropharia hornemannii</i> FBCC 565 (K122)	II
<i>Suillus bovinus</i> FBCC 1392 (JH95)	II
<i>Suillus variegatus</i> FBCC 1390 (JH35)	II

4.2 Overview of the most important methods

The experimental set-ups as well as the methods used are described in detail in **Publications I-III**. An overview of the most important methods is given in Table 3. The working principle of the bioluminescent yeast assays will be described in detail in paragraphs 5.1.2 and 5.1.3 and visualized in Figure 6. Additionally the protocol is given in Table 4. The extraction procedure of extracellular enzymes from agar plugs will be described in detail in paragraph 5.2.2 and visualized in Figure 7.

Table 3: Most important methods used in this work.

Method	Described in
Construction of <i>Saccharomyces cerevisiae</i> BMAEReluc/hRXR-ER	I
Monitoring of endocrine disrupting activity	
• interaction with RXR (in liquid and in sludged sediment)	I, Table 4
• interaction with ER α (in liquid)	III, Table 4
Chemical analysis of BPA and possible metabolites	III
Cultivation of fungi	
• on agar plates	II
• in liquid cultures	III
Preparation of enzymes	
• extraction from agar plugs	II
• purification of a neutral manganese peroxidase (MnP) from <i>S. coronilla</i>	III, (Steffen <i>et al.</i> 2002b)
Determination of enzyme activities	
• coupled assay for the detection of laccase and peroxidase	II, III
• enzyme fingerprint (determination of 12 different enzymes)	II
Conversion of BPA in liquid fungal cultures and with purified neutral MnP	III

Table 4: Yeast assay protocols for detection of estrogenic compounds based on Leskinen et al. (2005) and RXR ligands (Publication III) in liquid samples.

Step	Detection of estrogenic compounds	Detection of RXR ligands
Overnight culture of yeast	<ul style="list-style-type: none"> Cultivation of the reporter strain <i>S. cerevisiae</i> BMAEReluc/ERα in SC medium^{a)} supplemented with glucose and the amino acids histidine, leucine and alanine Cultivation of the control strain BMA64/luc in SC medium supplemented with glucose and the amino acids histidine, leucine, alanine, and tryptophane 	<ul style="list-style-type: none"> Cultivation of the reporter strain <i>S. cerevisiae</i> BMAEReluc/hRXR-ER in SC medium supplemented with galactose and the amino acids histidine, leucine and alanine Cultivation of the control strain BMA64/luc in SC medium supplemented with glucose and the amino acids histidine, leucine, alanine, and tryptophane
Incubation until culture reaches exponential growth phase is reached	<ul style="list-style-type: none"> Dilution of the overnight culture to OD₆₀₀ = 0.4 Cultivation in SD medium^{b)} using the same supplements as above Incubation at 30 °C while shaking until OD₆₀₀ = 0.6-0.7 is reached (2.5 h) 	<ul style="list-style-type: none"> Dilution of the overnight culture to OD₆₀₀ = 0.4 Cultivation in SD medium using the same supplements as above Incubation at 30 °C while shaking until OD₆₀₀ = 0.6-0.7 is reached (3 h)
Robot-assisted pipetting	<ul style="list-style-type: none"> Pipetting of 10 μl of sample and 90 μl of yeast culture using Biomek NX^P Laboratory Automation Workstation (Beckman Coulter, USA) to a 96-well plate 	<ul style="list-style-type: none"> Pipetting of 5 μl of sample and 45 μl of yeast culture containing 0.5 mM D-luciferin using Biomek NX^P Laboratory Automation Workstation (Beckman Coulter, USA) to a 384-well plate
Incubation	<ul style="list-style-type: none"> Brief shaking Incubation at 30 °C for 2.5 h 	<ul style="list-style-type: none"> Brief shaking Incubation at 30 °C for 3-5 h
Measurement of luminescence	<ul style="list-style-type: none"> Addition of 100 μl 1 mM D-luciferin Brief shaking Measurement of luminescence for 1 s/well using Victor³ 1420 Multilabel Counter (PerkinElmer Wallac, USA) 	<ul style="list-style-type: none"> Brief shaking Measurement of luminescence for 1 s/well using Victor³ 1420 Multilabel Counter (PerkinElmer Wallac, USA)

^{a)} SC medium: Difco yeast nitrogen base without amino acids and Difco yeast synthetic dropout mix without leucine, histidine, uracil and tryptophane (Beckton Dickinson)

^{b)} SD medium: Difco yeast nitrogen base without amino acids (Beckton Dickinson)

5 The fungal toolbox

5.1 Bioluminescent yeast assays as a reporter tool for endocrine disrupting activity

5.1.1 Bioreporter

Bioreporters are living organisms that produce a phenotypic reaction upon contact with a target chemical. The presence of organotin compounds, for example, is reported by gastropods as the development of imposex (Gibbs & Bryan 1986, Gibbs *et al.* 1990, Oehlmann *et al.* 1996, Oehlmann *et al.* 2007, Titley-O'Neal *et al.* 2011). Estrogenic compounds can be monitored in male fish through elevated levels of vitellogenins. Vitellogenins are egg-yolk protein precursors and should not be present in male and immature females (Matozzo *et al.* 2008). Furthermore cell-based assays can be employed as bioreporter tools. The latter approach reduces the need of animal experiments and it reduces experimental time and usually the amount of target chemicals as well. As an example, proliferating human breast tumor, MCF₇, cells incidentally reported the leaching of an estrogenic compound from polystyrene laboratory ware, which was identified as nonylphenol (Soto *et al.* 1991).

Microbial cells play an increasingly important role in the development of bioreporter assays, especially in combination with molecular biology tools. Specific assays can be engineered, typically by introducing a sensor element and a reporter gene as plasmid or directly into the genome of the bioreporter species (Jansson *et al.* 2000). Easy-to-handle and quickly growing microbes are especially well-suited to serve as bioreporters. Using microbes has another advantage: aseptic handling is not an absolute necessity as in the case, for example, with the handling of mammalian cells. Thus, “real” environmental samples can be applied in the assay. In contrast to chemical analysis, bioreporters monitor the bioavailable and bioactive potential rather than the total concentrations of a pollutant (Harms *et al.* 2006). They can indicate a potential risk of exposed organisms and thus, resemble animal experiments to some extent. Moreover, microbial bioreporters allow conclusions about the concentration of contaminants that are bioavailable for biodegradation (Harms 2011).

5.1.2 Bioluminescent yeast assay to detect estrogenic compounds

Several assays have been developed for the monitoring of endocrine disrupting compounds, which are based on the interaction with a nuclear receptor, such as the estrogen receptor alpha (ER α), and subsequent expression of a reporter gene including those encoding β -galactosidase (Routledge & Sumpter 1996, Svobodová & Cajthaml 2010, Wagner & Oehlmann 2009), green fluorescent protein (Bovee *et al.* 2004) and luciferase (Leskinen *et al.* 2003, Sanseverino *et al.* 2005). Especially yeast assays have been used to monitor estrogenic

activity in wastewater effluents (Salste *et al.* 2007), municipal waste landfill leachates (Coors *et al.* 2003), surface waters (Beck *et al.* 2006, Chu *et al.* 2009), and for testing new potentially harmful substances such as new variants of brominated flame retardants meant to replace phased-out predecessor compounds (Ezechiáš *et al.* 2012).

The bioluminescent yeast assay developed by Leskinen *et al.* (2005) for the detection of estrogenic compounds using *Saccharomyces cerevisiae* BMAEReluc/ER α as the reporter strain was a very important method used in **Publication III**. This specific strain expresses the human estrogen receptor alpha (ER α) from plasmid pG1/ER(G), constitutively. Upon binding a ligand, either bisphenol A or an estrogenic degradation product, the receptor dimerizes, binds to the estrogen response elements (ERE) and induces transcription of the *Photinus pyralis* (firefly) luciferase gene (*luc*) (Figure 6). Since the peroxisomal targeting codons have been removed, the substrate D-luciferin can be added to living cells and light emission can be measured without cell disruption (Leskinen *et al.* 2003). The bioluminescent yeast assay was performed in a 96-well plate format. Thus, it allows rapid analysis of small sample volumes (10 μ l) and provides results within one working day (Table 4). The emitted light intensity measured with a luminometer was used as the indicator for the estrogenic activity of compounds present in the sample; the higher the light intensity the higher the estrogenic activity.

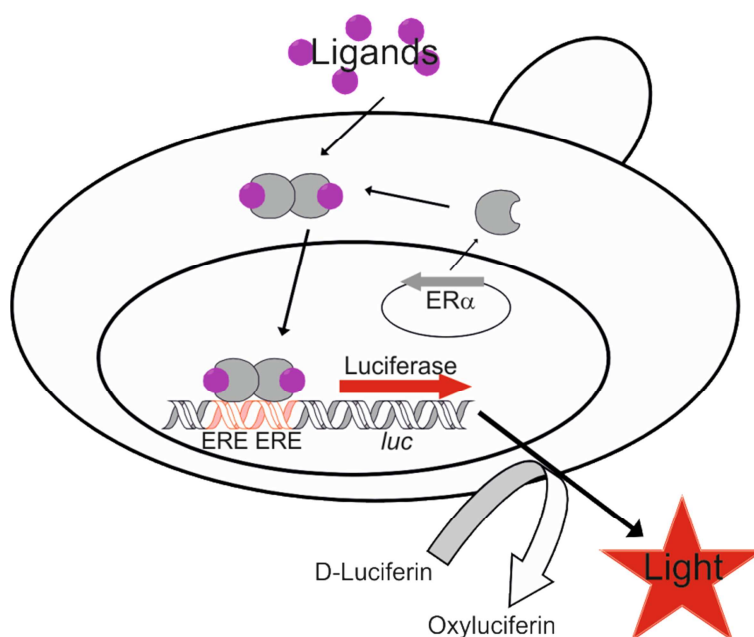


Figure 6: Principle of the bioluminescent yeast assay to detect estrogenic compounds. ER α : human estrogen receptor alpha, ERE: estrogen response element, *luc*: *Photinus pyralis* (firefly) luciferase gene. (Scheme drawn by Grit Kabiersch and Johanna Rajasärkkä)

The bioluminescent yeast assay has the potential to be adapted to answer varying or newly developing questions. Thus, the assay procedure was miniaturized into high-throughput 384- and 1536-well plate formats (Rajasärkkä & Virta 2011). Furthermore, the target specificity can be changed; the ER α was modified in such a way that it specifically binds to BPA (Rajasärkkä *et al.* 2011). Another approach is to exchange the target receptor in order to detect other EDCs, such as dioxins and dioxin-like compounds (Leskinen *et al.* 2008). Last but not least, the target receptor can also be exchanged to detect virilizing organotin compounds, which led to **Publication I**.

5.1.3 Bioluminescent yeast assay to detect organotin compounds (Publication I)

Chemical analysis of organotin compounds requires sensitive and laborious methods such as gas chromatography, accompanied with large sample volumes and large volumes of hazardous extraction and derivatization chemicals (Binato *et al.* 1998, Ikonomou *et al.* 2002). Microbial bioreporters help to overcome these drawbacks since they are easy-to-handle, sample preparation is usually less laborious, smaller sample volumes and less hazardous liquids are needed. Additionally, bioavailability and thus, a potential risk towards target organisms can be estimated. In **Publication I**, a yeast assay was constructed and characterized that can be employed to detect organotin compounds in liquid and sediment samples based on interaction with the retinoid X receptor (RXR) and subsequent expression of a reporter luciferase.

The working principle is very similar to the bioluminescent yeast assay for estrogenic compounds, except for the introduction of a different target receptor, the RXR. A chimeric human retinoid X receptor alpha (hRXR-ER) was constructed, which contains the ligand-binding domain of RXR and the DNA-binding domain of the human ER α . Consequently, this receptor interacts with typical ligands of RXR but the modification of the DNA-binding domain allows the use of the reporter strain BMAEReluc as a host, in which the luciferase gene is under the control of estrogen responsive elements (ERE) (Figure 1 in **Publication I**). Expression of hRXR-ER is galactose-inducible and the assay was carried out in a 384-well plate format, which resulted in small changes of the protocol (Table 4).

TBT could be detected at concentrations as low as 60 nM (Figure 2 in **Publication I**). The assay demonstrated to also be specific for related organotin compounds and natural ligands of the retinoid X receptor with the following potencies: tributyltin \geq 9-*cis* retinoic acid \geq triphenyltin > diphenyltin > dibutyltin \geq monophenyltin \geq all-*trans* retinoic acid. This order is in line with observations from receptor activation studies carried out by others (Nishikawa *et al.* 2004). Monobutyltin as well as two non-tin compounds, namely bisphenol A and benzo[a]pyrene, could not be detected using this approach (Figure 3 in **Publication I**).

In order to test the applicability of this assay for environmental samples, a mixture of tributyltin and dibutyltin was applied. It turned out that DBT had only a minor effect on the overall endocrine disrupting activity in real samples (Figure 4 in **Publication I**). Finally, a true

sediment sample spiked with TBT was investigated and revealed that the assay was suitable for turbid solutions, such as sludged sediments, but that the sensitivity was lower compared to clear solutions (Figure 5 in **Publication I**).

In addition to the yeast-based assay described in this work, other microbial bioreporter systems for the detection of organotin compounds exist. Durand *et al.* (2003) describe a recombinant luminescent bacterial assay, which detects TBT with similar sensitivity and DBT at very low concentrations starting from 0.1 nM. Triphenyl compounds could not be detected. This assay does not reflect natural toxicity but it might be a useful tool to monitor the progress of TBT degradation to DBT and further on to MBT. Furthermore, the mechanism of induction is unknown. The yeast two-hybrid assay described by Nishikawa *et al.* (2004) also detects TBT at nanomolar concentrations. However, this assay uses β -galactosidase as a reporter, which requires longer incubation times and is thus less suitable for high-throughput purposes.

To summarize, the most important achievements of the bioluminescent yeast assay for the detection of organotin compounds were:

- The assay was highly specific for organotin compounds and natural ligands of the retinoid X receptor.
- It detected tributyltin and triphenyltin in nanomolar concentrations.
- The assay allowed small-scale, high-throughput analysis and cost-efficient monitoring of environmental samples.
- Results were provided within one working day.
- Bioavailability as well as mixture effects of different organotin compounds could be predicted.
- This is the first RXR-based bioluminescent yeast-assay characterized for the use of environmental samples.
- The assay was less sensitive with sludged sediment compared to clear solutions.

5.2 Litter-degrading fungi as a degradation tool for endocrine disrupting compounds

5.2.1 Enzymatic potential of litter-degrading fungi for the degradation of xenobiotic compounds

Fungi used in this work are litter-degrading fungi that have the potential to degrade xenobiotic compounds. Litter-degrading fungi preferably colonize grasslands and the upper most soil layer. Like basidiomycetous wood-rotting white-rot fungi, many litter-decomposing fungi produce extracellular enzymes with low substrate specificity (Hatakka & Hammel 2010, Hatakka 1994), enabling them to modify and degrade the lignin part of the plant litter material.

The major components of plant cell walls are cellulose, hemicelluloses and lignin. Cellulose is composed of D-glucose units, which are linked by $\beta(1-4)$ -glycosidic bonds and form a linear polymer. Hemicellulose polymers are shorter than cellulose chains. They are frequently branched and contain several sugars, of which D-xylans and D-mannans are the most important (Chauhan *et al.* 2012, Jordan *et al.* 2012). Lignin is closely associated with cellulose and hemicellulose; it protects the plant cell wall from microbial attack and provides rigidity to the plant (Leonowicz *et al.* 1999, Martínez *et al.* 2005). It is a highly branched, irregular three-dimensional polymer composed of guaiacyl, syringyl and *p*-hydroxyphenyl subunits, linked via diverse carbon-carbon and ether bonds due to radical coupling reactions (Martínez *et al.* 2005). Consequently, the lignin molecule contains aromatic, furanoid, dibenzodioxocin and propanoid structures as well as a variety of functional groups such as phenolic hydroxyl, alcoholic hydroxyl, aldehyde groups and methoxyl side chains (Brunow 2005). The most important enzymes for the degradation of lignin are laccase (EC 1.10.3.2) and the peroxidases such as manganese peroxidase (MnP, EC 1.11.1.13), lignin peroxidase (EC 1.11.1.14) and versatile peroxidase (EC 1.11.1.16).

Laccases are multicopper glycoproteins with a carbohydrate content varying between 10 and 25 %, typical molecular masses are between 60-70 kDa, isoelectric points are at around pH 4.0 and the broad pH optimum ranges from less than 2.0 to 7.0, depending on the substrate (Baldrian 2006). They catalyze the oxidation of a variety of phenolic and other low redox potential compounds through a complete four-electron reduction of molecular oxygen to water (Call & Mücke 1997, Hatakka & Hammel 2010). The four conserved copper ions classified as one type-1 (T1), one type-2 (T2) and two type-3 (T3) coppers in the active site are involved with the catalysis. Substrates are oxidized near the T1 copper and the electrons are transferred through an internal electron transfer mechanism to the trinuclear T2/T3 copper cluster (Piontek *et al.* 2002, Shleev *et al.* 2005).

Manganese peroxidase (MnP), lignin peroxidase and versatile peroxidase are extracellular glycoproteins with an iron protoporphyrin IX (heme) prosthetic group. They require H_2O_2 for catalysis. The oxygen-oxygen bond of H_2O_2 is cleaved and two electrons are transferred, resulting in the formation of a Fe^{4+} -oxo-prophyrin-radical complex (Compound I). In the case of manganese peroxidase, a monochelated Mn^{2+} acts as a one-electron donor and is oxidized to Mn^{3+} , which is subsequently chelated by organic acids such as oxalate. During this reaction, Compound I is reduced and, consequently, forms the Fe^{4+} -oxo-porphyrin complex (Compound II). Compound II is further reduced to the initial state and another Mn^{2+} is oxidized to Mn^{3+} . The chelated Mn^{3+} acts as a low molecular mass, diffusible redox-mediator, which oxidizes aromatic compounds via hydrogen abstraction. Ultimately, this results in the formation of radicals, which attack recalcitrant structures (Hofrichter 2002). In the case of lignin peroxidase, veratryl alcohol acts as electron donor, redox-mediator and radical generator (Hatakka & Hammel 2010). Versatile peroxidases combine the catalytic properties of manganese peroxidase and lignin peroxidase, since they can oxidize both Mn^{2+} and veratryl alcohol (Ruiz-Dueñas *et al.* 2009). Recently, two other heme-containing

oxidoreductases arrived on the scientific scene: manganese-independent dye-decolorizing peroxidases (EC 1.11.1.19) (Liers *et al.* 2010) and unspecific peroxygenases (EC 1.11.2.1) (Hofrichter & Ullrich 2006, Peter *et al.* 2013), both of which are secreted by fungal cultures growing on beech wood and suggest an involvement of these enzymes in the conversion of lignin or lignin metabolites (Liers *et al.* 2011). Furthermore, other oxidative enzymes such as glyoxal oxidase (EC 1.2.3.5), aryl alcohol oxidase (EC 1.1.3.7), pyranose 2-oxidase (EC 1.13.10) and glucose 1-oxidase (EC 1.1.3.4), depending on the fungus, generate hydrogen peroxide, required by the peroxidases and thus, serve as accessory enzymes in the lignin degradation process (Hatakka & Hammel 2010). However, the most important enzymes produced in litter-degrading fungi are laccase and manganese peroxidase.

The above described lignin-degrading enzymes have broad substrate specificity and also possess the capability to oxidize several environmental pollutants (Reddy 1995, Tuomela & Hatakka 2011). Laccases can be used for environmental purposes such as dye decolorization or bleaching of effluents from the pulp and paper industry (Barneto *et al.* 2012, Daâssi *et al.* 2012, Mendoza *et al.* 2011, Nitheranont *et al.* 2011). They can be heterologously expressed in other organisms, for example, in *Pichia pastoris* (Hildén *et al.* 2013) or *Aspergillus oryzae* (Berka *et al.* 1997) and thus, can be produced in large scale and commercially (Østergaard & Olsen 2010). In general, laccases would require only oxygen for catalysis; however, in many cases small molecular mass redox mediators such as 1-hydroxybenzotriazole (HBT) are needed and used in order to enlarge the substrate spectrum (Cañas & Camarero 2010, Majeau *et al.* 2010). The redox mediators may pose an additional environmental risk, i.e. HBT has been shown to be toxic (Suda *et al.* 2012, Valls *et al.* 2012), and is counterproductive for the usage of laccase in eco-friendly processes. Also natural-type compounds, originating from lignin substructures, have been tested for their potential as mediators (Nousiainen *et al.* 2009) but usually they are not as efficient as HBT. For this reason, attention is also directed to peroxidases, which bear enormous potential for the degradation of pollutants (Liers *et al.* 2013, Qayyum *et al.* 2009). Experiments with partially purified manganese peroxidases in cell-free systems supplied evidence that aromatic compounds such as benzo[a]pyrene (Steffen *et al.* 2003) or aminonitrotoluenes (Scheibner & Hofrichter 1998) can be converted and mineralized. However, the use of purified enzymes for bioremediation purposes requires large quantities of these enzymes accompanied with some effort to optimize the production process (Babič & Pavko 2012, Babič *et al.* 2012). Additionally, enzymes need to be stabilized and immobilized in order to reduce deactivation (Cabana *et al.* 2009, Cao *et al.* 2003, Cao 2005, Corvini & Shahgaldian 2010, Modaressi *et al.* 2005).

Bioremediation comprises a large field of technologies, including microbiology, chemistry, geology and engineering. Bioremediation strategies range from monitoring of degradation processes without interference (monitored natural attenuation) to the improvement of environmental conditions for degrading organisms in the contaminated area (enhanced natural attenuation) and even further to inoculation of contaminated matrices (soil or water)

with organisms known to degrade the pollutant (bioaugmentation) (Jørgensen 2011). Using whole fungal cultures as degradation tools provides the advantage that those enzymes described above may be produced and secreted continuously by viable cultures even under nutrient-deficient conditions (Pointing 2001, Reddy 1995). Lignin-degrading enzymes are produced during secondary metabolism. In contrast to pollutant-specific bacteria, litter-degrading fungi do not use these pollutants as single carbon energy, which allows efficient degradation also when contaminants are present in low concentrations. Fungi require additional carbon sources for growth, ideally lignocellulosic material such as bark, flax or straw (Steffen *et al.* 2007, Valentín *et al.* 2009), which are inexpensive and can be added easily to contaminated soil (Steffen & Tuomela 2010, Winkvist *et al.* 2009). Litter-degrading fungi grown on such lignocellulose carrier materials were demonstrated to grow into soil and to degrade polycyclic aromatic hydrocarbons (Steffen *et al.* 2007) or trinitrotoluene (Herre *et al.* 1998). In solid media, fungi grow by hyphal extension. This characteristic allows penetration of a given substrate in a way that bacteria are unable to (Reddy 1995), enlarging the fungi's spatial degradation potential. Fungal hyphae have also been demonstrated to facilitate bacteria translocation and, through it, increase bioavailability of pollutants for bacteria, improving the overall degradation potential of polycyclic aromatic hydrocarbons in concerted action (Kohlmeier *et al.* 2005, Wick *et al.* 2007).

A successful bioremediation experiment requires thorough planning of all parameters. Thus, a careful screening strategy is necessary that takes into account ecophysiological characteristics such as toxicity of the contaminant, additional contamination of the target area with heavy metals, salinity, nutrient availability, synergistic or antagonistic effects of endogenous microflora and bioaccessibility of the contaminant (Harms 2011, Jørgensen 2011, Steffen & Tuomela 2010, Tuomela *et al.* 2005, Valentín *et al.* 2006, Valentín *et al.* 2007).

5.2.2 Novel sampling method to screen for enzyme production in solid media (Publication II)

Determination of enzyme activities in solid media usually goes hand in hand with destructive harvesting, large sample volumes, laborious extraction procedures or addition of large volumes of water or buffer (Dinis *et al.* 2009, Elisashvili *et al.* 2008, Hahn *et al.* 2013, Isikhuemhen *et al.* 2009, Steffen *et al.* 2007, Valentín *et al.* 2010, Šnajdr & Baldrian 2006) and, consequently, strong dilution effects. In this study, a novel sampling method was developed that allows non-destructive, fine-scale sampling, employing a fungal culture growing in solid agar medium.

A circular agar plug with a diameter of 8 mm and an approximate volume of 250 µl was cut out using a cork borer (Figure 7A). The plug was placed into a Costar® Spin-X® CLS8162 (Corning Inc., NY, USA) centrifuge tube (Figure 7B). This filter consists of a cellulose acetate membrane with a pore size of 0.45 µm. A volume of approximately 150-200 µl of solution

was obtained after centrifugation at 20 °C at 15,700 x g for 30 min (Figure 7C). This volume was used for the determination of extracellular enzyme activities as described in **Publication II**.

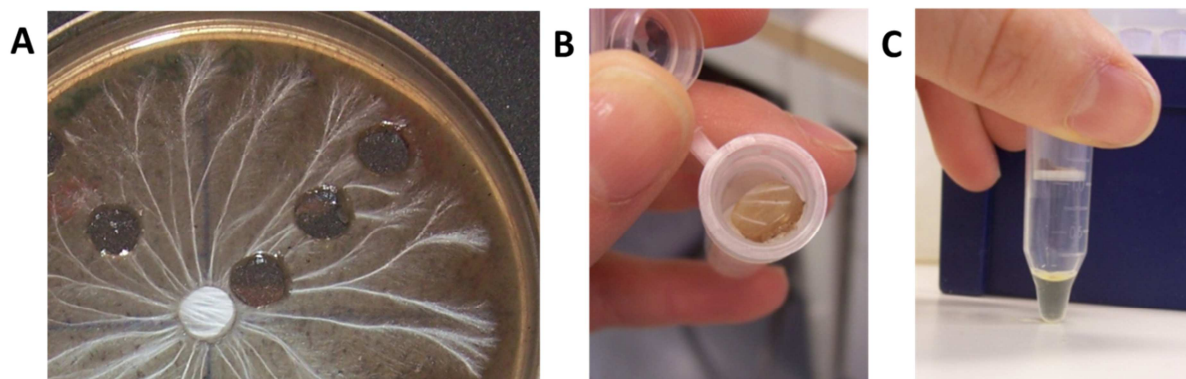


Figure 7: Sampling procedure. (A) *Agrocybe praecox* on spent coffee ground agar. (B) An 8 mm circular agar plug was placed into the filter tube. (C) 150-200 µl of liquid was obtained after centrifugation.

Centrifugation recovered the liquid trapped in the sample, which contained water extractable material, including many extracellular enzymes. The recovery of two added model enzymes, laccase and MnP, ranged from 50 to 75 % (Figure 1 in **Publication II**) for most of the tested media. Addition of humic substances decreased recovery of MnP to 25 %. This loss should be taken into account in applications involving soil or other organic additives (Table 2 in **Publication II**).

The novel method allowed the visualization of the spatial distribution of laccase and peroxidase activities of the litter-decomposing fungus, *Agrocybe praecox*, on agar plates, containing spent coffee ground as a solid growth substrate, and plotting against the calculated age of the mycelium in a non-destructive manner (Figure 4 in **Publication II**). This simple experiment clearly demonstrated that the effect of additives on growth and enzyme production can be studied in a single experimental set-up. This can be especially useful in screening approaches for efficient degraders of environmental pollutants. Furthermore spatial data can be obtained, which might be of importance when studying ecological systems and interactions. Hahn *et al.* (2013) recently developed an experimental setup to study the spatiotemporal degradation of wood in fungal microcosms. The assay is performed in silicon tubes with varying growth parameters. The fungus grows unidirectionally and segments of it are analyzed after the experiment is terminated. This experimental approach is very well-suited for simulation studies, whereas, in comparison, the experimental set-up described in **Publication II** is non-destructive and very well suitable for quicker screening approaches.

The most important advantage of the method described in **Publication III** is that laborious extraction procedures and addition of liquids becomes unnecessary, provided that the culture contains enough moisture to be separated as a liquid through centrifugation. This prevents dilution of extracted enzymes below the detection limit. Consequently, the method enables better detection of low enzyme activities, allowing a comprehensive determination of functional enzyme finger prints (Table 3 in **Publication II**).

To conclude, this method has proven to be applicable for:

- Small-scale screening approaches, i.e. determination of functional enzyme finger prints or effects of added chemicals such as environmental pollutants on fungal growth and enzyme production.
- Determination of spatial distribution of enzyme activities in micro- and mesocosms.
- Investigation of the conversion of toxic, colorless additives in combination with other small-scale analysis tools, i.e. bioluminescent yeast assays.

5.3 Degradation of bisphenol A and complete removal of the estrogenic activity (**Publication III**)

The aim of this study was to investigate the extent, to which the endocrine disrupting compound bisphenol A was degraded *in vivo* by cultures of two litter-decomposing fungi, namely *Stropharia rugosoannulata* and *Stropharia coronilla*, and to demonstrate the complete removal of the estrogenic effect using a bioluminescent yeast assay, containing the estrogen receptor alpha. In both cultures, estrogenic activity was successfully and completely removed within 24 h (Figure 1 and 2 in **Publication III**).

S. coronilla produces a MnP with nearly neutral isoelectric point (pI around 6.3-7.1) (Steffen *et al.* 2002b). This enzyme has the potential to function even at slightly alkaline pH values, whereas other MnPs are usually active in more acidic environments (Hofrichter 2002). In order to further explore this potential, the neutral MnP of *S. coronilla* was purified and employed for BPA degradation in a cell-free *in vitro* reaction system, containing Mn^{2+} and malonate as chelator. BPA concentration, monitored by high performance liquid chromatography (HPLC), decreased rapidly. Within 90 min approximately 98 % of the initial BPA concentration had been transformed. Parallel determination of the estrogenic activity revealed that also the estrogenic effect was removed concomitantly (Figure 3 in **Publication III**). During the reaction, yellow-brown precipitates were observed indicating the formation of insoluble polymerization products, which was confirmed by the detection of dimers (MW 454) and trimers (MW 670) by liquid chromatography-mass spectrometry (LC-MS) (Figure 4 in **Pulication III**). Peroxidases (and also laccases) are well-known for catalyzing oxidation of phenolic substrates (such as BPA) into phenoxyl radicals, which, in turn, may couple to form oligomeric or polymeric products (Mao *et al.* 2010, Uchida *et al.* 2001). Such enzymatic coupling reactions play an important role in detoxification of

contaminants, since an increasing size of coupling products reduces bioavailability (Weber & Huang 2003) and, in the case of BPA coupling products, decreases the ability of interacting with estrogen receptors.

A closer look at laccase and MnP activities upon contact with bisphenol A revealed that enzyme activities were strongly affected, especially in the cell-free reaction (Figure 3 in **Publication III**). Degradation studies with BPA, using laccases, demonstrated that enzyme life times and activities could be extended when adding stabilizers such as polyethylene glycol (PEG) to the reaction solution (Modaressi *et al.* 2005) or by immobilization techniques (Cabana *et al.* 2007, Demarche *et al.* 2012). In this study, activities of laccase and MnP recovered in fungal cultures, since they were secreted continuously during further cultivation (Figures 1 and 2 in **Publication III**).

Degradation or chemical transformation of BPA has been reported for a number of microorganisms, including bacteria, and lignin-degrading fungi. One reported side effect of bacterial degradation is the formation of intermediates with higher estrogenic potential than that of the parental compound bisphenol A (Li *et al.* 2012, Oshiman *et al.* 2007). After treatment with lignin-degrading fungi such as *Stereum hirsutum*, *Heterobasidion insulare* or *Pleurotus ostreatus*, only small amounts of residual BPA were reported (Hirano *et al.* 2000, Lee *et al.* 2005). In this study, estrogenic activity of BPA was removed rapidly and permanently in cultures of *S. coronilla*. In cultures of *S. rugosoannulata*, however, estrogenic activity re-appeared (Figure 1 in **Publication III**). Although this estrogenic activity amounted to only 5 % of the initial activity, this observation gave rise to further analysis of possible metabolites. LC-MS analysis of the estrogenic culture liquid extract revealed compounds with molecular masses of 220 and 236, which point to metabolites of BPA with one intact hydroxyphenyl group that still allows interaction with estrogen receptors (Figure 5 in **Publication III**).

Finally, a hypothetical pathway for BPA degradation was proposed (Figure 6 in **Publication III** and Figure 8). Phenoxyl radicals are generated during one-electron oxidation of BPA by the action of extracellular MnP/Mn³⁺ or laccases (Baldrian 2006, Hofrichter 2002). These phenoxyl radicals may undergo oxidative coupling reactions in order to form polymerization products. Phenoxyl radicals may also react with superoxide, which is formed when Mn³⁺ decarboxylates carboxylic acids to alkyl radicals. Upon this reaction, unstable ether peroxides form, which spontaneously cleave to give ring fission products. Subsequent decarboxylation and hydrogenation reactions *in vivo* result in an aldehyde compound.

In addition to degradation of BPA by extracellular fungal oxidative enzymes, BPA can also be converted by intracellular cytochrome P450 monooxygenases (Subramanian & Yadav 2009). Lignin-degrading fungi possess an exceptionally high number of P450 genes (Floudas *et al.* 2012, Martinez *et al.* 2004), indicating that these types of enzymes most probably play a role in the degradation of various compounds with benzene ring structures. Alternatively, extracellular unspecific peroxygenases have the potential to hydroxylate a variety of

compounds (Kluge *et al.* 2012, Peter *et al.* 2011), and they exist as hypothetical genes in litter-decomposing fungi such as *Agrocybe praecox* and *Hebeloma cylindrosporum*, both belonging to the family of Strophariaceae. The resulting product, containing a hydroxylated methyl group (Kolvenbach *et al.* 2007, Sasaki *et al.* 2005), is likely to undergo the same chemical transformation as BPA, giving rise to a ring fission product with an aldehyde group and one remaining intact hydroxyl group. Due to the intact hydroxyl group, the latter proposed metabolites are still able to interact with the estrogen receptor alpha in the bioluminescent yeast assay and may account for the re-emerging estrogenic activity in cultures of *S. rugosoannulata*. Similar metabolites have been observed in a very recent publication by Ding *et al.*, in which degradation of the flame retardant compound tetrabromobisphenol A was induced with sulfate radicals (Ding *et al.* 2013).

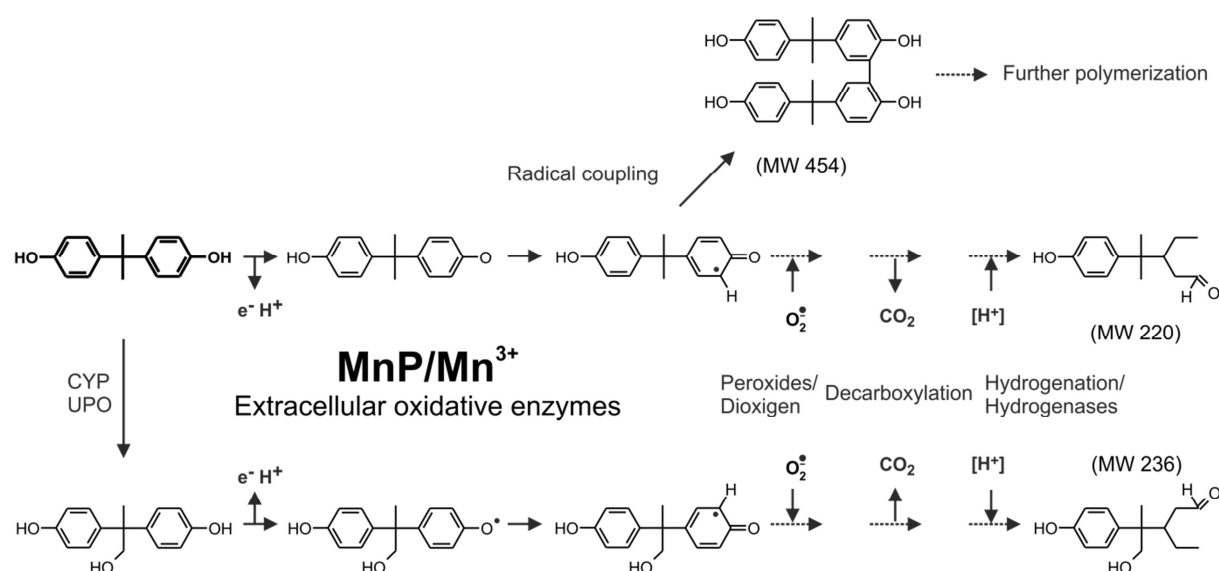


Figure 8: Hypothetical reaction pathways of BPA in the presence of oxidative enzymes. CYP = cytochrome P450 monooxygenase system, UPO = extracellular unspecific peroxygenases, MnP = manganese peroxidase, MW = molecular weight. (Adapted from Publication III)

To conclude,

- Estrogenic activity of bisphenol A could be removed rapidly and permanently. Here *S. coronilla* proved to be the most efficient fungus.
- Treatment of BPA with litter-degrading fungi and their oxidative enzymes resulted in oxidative coupling products, which reduced bioavailability of BPA and, consequently, estrogenic activity.
- Transient metabolites might be formed during degradation and these may have estrogenic activity, although it was considerably lower than the initial activity of BPA.

- The bioluminescent yeast assay was a valuable monitoring system for estrogenic activity that specifically pointed to samples that needed further chemical characterization.

5.4 Strategy for rapid screening and upscaling

Lignin-degrading fungi produce highly active oxidative enzymes that have the capability of decomposing recalcitrant substances, such as lignin. Fungal oxidative enzymes also have the potential to degrade a variety of man-made hazardous chemicals, including polycyclic aromatic hydrocarbons (Steffen *et al.* 2002a) or pentachlorophenol (Tuomela *et al.* 1999) as well as endocrine disrupting compounds such as bisphenol A (**Publication III**) and organotins (Bernat & Długoński 2002). However, in order to tap the full potential of fungi and their oxidative enzymes, special attention should be paid to ecological demands (Harms *et al.* 2011). Based on the methods developed and experience gathered throughout the doctoral work, a strategy was developed for the rapid screening of efficient pollutant-degrading organisms, suitable for the upscaling of bioremediation attempts (Figure 9). In a single experimental set-up, employing nothing more than agar plates, toxicity tests, identification of efficient extracellular enzyme producers and successful removal of endocrine disrupting activity can be combined. Fungi may grow on agar plates supplemented with the pollutant or may be put directly onto polluted environmental material.

As a simple preliminary test experiment, malt agar plates were overgrown with *Agrocybe praecox*. Some parts of the plate were cut out and filled with sediment spiked with different concentrations of tributyltin. The potential to grow into the sediment, tolerate the pollutant but also competition with indigenous microorganisms can be observed visually (Figure 9). As a next step, using the sampling method described in **Publication II**, spatial distribution of extracellular enzyme activities can be determined as well as the effect of the pollutant on enzyme production. Furthermore, from these spots the endocrine disrupting activity can be determined using a bioluminescent yeast assay displaying target receptors as developed in **Publication I** or used in **Publication III**. The most promising candidates from these screening attempts can be further employed for degradation studies, which should include, again, determination of enzyme activities, tracking of the removal of endocrine disrupting activity and, additionally, chemical analysis of selected samples for determination of metabolites, and a proposal of a degradation pathway and remaining risks as demonstrated in **Publication III**. Finally, the best candidate(s) could be selected and tested in pilot and large scale bioremediation attempts.

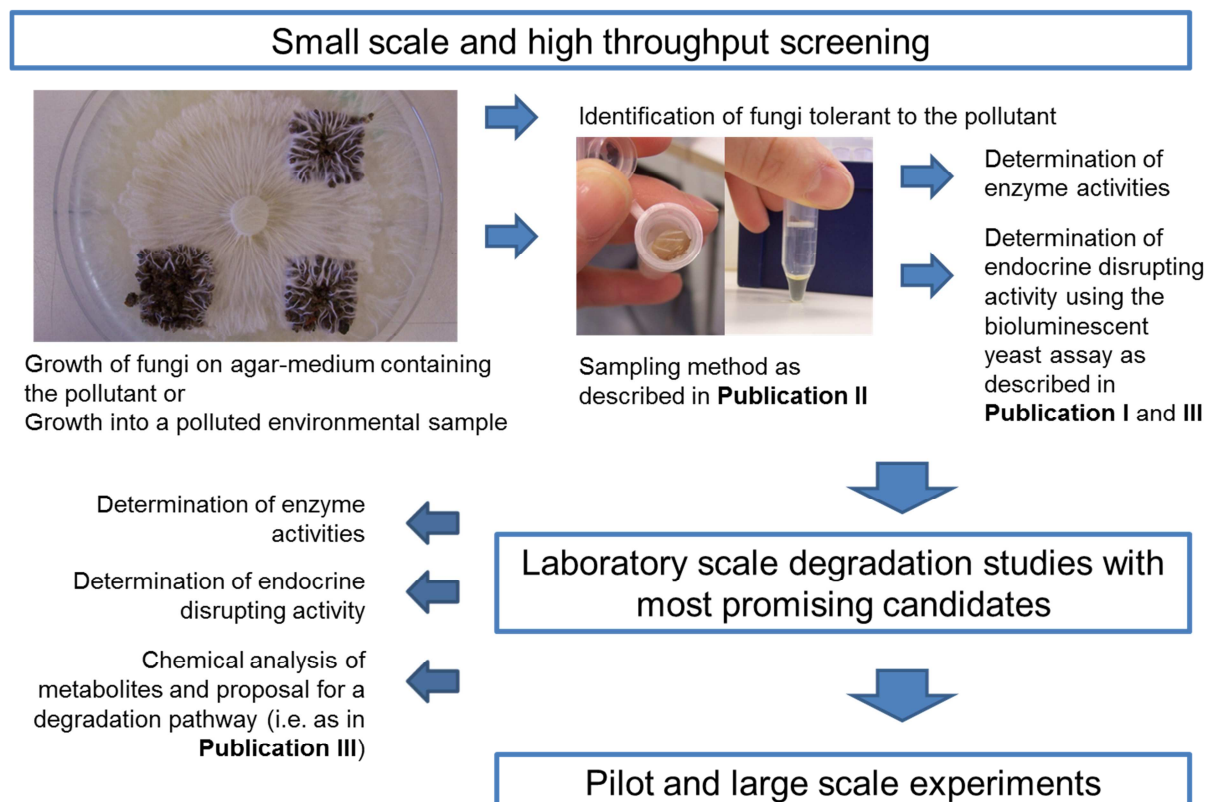


Figure 9: Identification of efficient, pollutant-degrading organisms – simple strategy from screening to large scale bioremediation experiments. The plate shows *Agrocybe praecox* growing into sediment samples spiked with different concentrations of tributyltin.

6 Conclusions

Endocrine disrupting compounds can be successfully degraded using the enzymatic potential of litter-degrading fungi. Bioluminescent yeast assays, containing specific target receptors, are valuable monitoring tools to follow the degradation and predict remaining risks.

Bioluminescent yeast assays allow reliable detection of compound groups and can be performed in small scale, cost-efficiently and in a high-throughput manner. Additionally, they allow further adaptations to detect other compound groups, which may come into focus in the future.

Litter-degrading fungi are a useful tool to alleviate toxic and endocrine disrupting effects. A careful screening strategy, taking ecological demands into account, is a prerequisite for tapping their full potential.

Applying both fungal tools, bioluminescent yeasts and lignin-degrading basidiomycetes, endocrine disrupting compounds can be degraded successfully and success can easily be followed. Chemical analyses cannot and should not be totally avoided, since metabolites and potential degradation pathways need to be evaluated. Nevertheless, the number of chemical analyses can be reduced, thus also decreasing the use of harmful extraction and derivatization chemicals in the laboratory.

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A handwritten signature in black ink, appearing to read "Grit Kärr". The signature is written in a cursive, flowing style. The first name "Grit" is on the left, and the last name "Kärr" is on the right, with a long, sweeping horizontal stroke extending from the end of the last name.

Helsinki, July 2013

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